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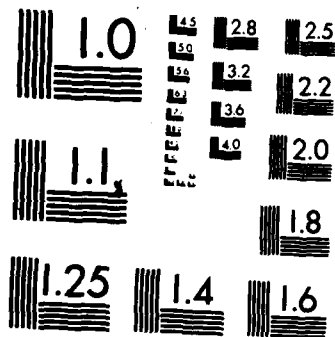UNCLASSIFIED   DEC 83 USAFSAM-TR-83-42 F33615-80-C-0612   F/G 6/18   NL

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EFFECTS OF LONG-TERM LOW-LEVEL RADIOFREQUENCY RADIATION EXPOSURE ON RATS

VOLUME 4. OPEN-FIELD BEHAVIOR AND CORTICOSTERONE

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December 1983

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NOTICES

This final report was submitted by the Bioelectromagnetics Research Laboratory, Department of Rehabilitation Medicine, School of Medicine, University of Washington, Seattle, Washington 98195, under contract F33615-80-C-0612, job order 7757-01-71, with the USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, Brooks AFB, Texas. Dr. Jerome H. Krupp (USAFSAM/RZP) was the Laboratory Project Scientist-in-Charge.
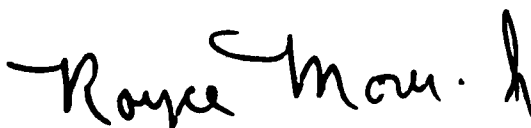
The animals involved in this study were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources - National Research Council.

The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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No significant alterations in overall behavioral response or corticosterone content were apparent in rats after 2 years of exposure to pulsed-microwave radiation. A reduction in open-field activity by the exposed animals during the first assessment period corresponded with an increase in serum corticosterone.

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EFFECTS OF LONG-TERM LOW-LEVEL RADIOFREQUENCY
RADIATION EXPOSURE ON RATS

VOLUME 4. OPEN-FIELD BEHAVIOR AND CORTICOSTERONE

INTRODUCTION

Although more than 6000 articles on the biological effects of electromagnetic wave radiation have been published, whether or not long-term low-level exposure to radiofrequency radiation (RFR) represents a human health hazard remains unclear (Czerski et al., 1974; Glaser and Dodge, 1977; Tyler, 1975; Justesen and Guy, 1977; Justesen and Baird, 1979; Gandhi, 1980). Most exposure protocols completed to date have been of relatively short duration and restricted sample size, thus providing little insight into cumulative effects.

During the past three years, the Bioelectromagnetics Research Laboratory at the University of Washington has conducted the largest single evaluation study of the bioeffects of microwaves ever undertaken. The goal of the project was to investigate purported adverse effects on health due to long-term exposure to pulsed-microwave radiation. The major emphasis was to expose a large population of experimental animals to microwave radiation throughout their lifetimes and monitor them for cumulative effects on general health and longevity.

A series of reports covers the conduct and results of each major aspect of the chronic exposure study. This technical report is the fourth; it treats in detail the periodic assessments of open-field behavior and corticosterone levels. The first three reports cover the exposure facility, animal maintenance procedures, and various dosimetry studies; the following paragraphs provide a brief summary of these topics.

A unique exposure facility was prepared that enabled 200 rats to be maintained under specific-pathogen-free (SPF) conditions while housed in individual, circularly polarized waveguides. The exposure facility consisted of two rooms, each containing 50 active-exposure and 50 sham-exposure waveguides to house subjects. Each room contained two 2450-MHz pulsed-microwave generators, each capable of delivering a maximum average power of 10 W at 800 pps, with a 10-µsec pulse width. This carrier

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was square-wave modulated at an 8-Hz rate. The power-distribution system delivered .144 W to each exposure waveguide for an average power density of 480 $\mu W/cm^2$. Analysis of whole-body calorimetry, thermographic data, and power-meter readings indicated that these exposure conditions resulted in average specific absorption rates (SARs) ranging from approximately 0.4 W/kg for a 200-g rat to 0.15 W/kg for an 800-g rat.

Two hundred male Sprague-Dawley rats were obtained from a commercial barrier-reared colony at 3 weeks of age and randomly assigned to exposed and sham-exposed treatment conditions. Exposure began at 8 weeks of age and continued for 25 months. Throughout this period, all surviving animals were bled at regular intervals and blood samples were analyzed for a panel of serum chemistries, hematological values, protein electrophoretic patterns, and thyroxine (T_4) and serum corticosterone levels. In addition, subpopulations of the exposed and sham-exposed populations were measured daily for body weight and food and water consumption, and periodically for oxygen consumption and carbon dioxide production. Activity in an open-field apparatus was assessed at regular intervals throughout the study. After 13 months, 10 rats from each treatment condition were killed for an interim evaluation consisting of immunological competence tests, whole-body analysis, and gross and histopathological examinations. At the end of 25 months, similar analyses were made of the surviving 10 rats from each group.

OPEN-FIELD BEHAVIOR

The sole behavioral endpoint included as part of the overall protocol for the long-term study was an assessment of open-field activity. It was chosen after a range of suggested behavioral tests--including shuttlebox avoidance, activity wheel, discriminated T-maze, and various schedule-reinforced, bar-pressing paradigms--had been evaluated according to the following criteria.

First, the behavioral test selected should not jeopardize the health of the animals and thus interfere with the primary goal of the project, i.e., evaluation of the status of health throughout life, and effects on mortality. Second, the test should not lead to obvious reactions to stress or differential experience (e.g., shock density) based on level of performance during testing. Third, the test must be easily performed within the confines of the SPF facility and the time schedule of the daily maintenance procedures. Fourth, the test must not be subject to bias on the part of the experimenter. Finally, the test must have a history of reported sensitivity in microwave-exposure studies.

The open-field test is not the most impressive of the behavioral test procedures considered, but it satisfies the selection criteria. It is simple in nature, does not rely on elaborate or time-consuming training procedures or shock-motivated performance, and can be routinely administered by laboratory personnel under the rigid SPF protocol.

Since its introduction nearly 50 years ago (Hall, 1934), the open-field test has become one of the most widely used behavioral test procedures, partly because of its simplicity (Archer, 1973; Walsh and Cummins, 1976). Animals are placed in a 3-ft square or round arena and observed for naturally occurring behaviors. The floor is often marked off into large black and white squares for quantification of ambulation. As many as 30 different behaviors have been measured, but the most frequently observed are ambulation, rearing, defecation, urination, and latencies. The open-field test has been used as a sensitive endpoint in many areas of research, including toxicology, pharmacology, and developmental biology.

East European researchers have used the open-field test extensively in studies of the bioeffects of microwaves. Their reports indicate that it is the most sensitive of the behavioral tests used, revealing complex

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relationships between observed behaviors, length of exposure, and power density (Shandala et al., 1979; Shandala and Markeev, 1980). United States researchers have reported similar relationships in open-field behaviors after microwave exposure (Lovely et al., 1978a).

Equipment and Methods

Apparatus

One problem historically associated with use of the open-field test was its susceptibility to bias on the part of the experimenter. As with many behavioral test procedures, in the open-fie.d test the experimenter places the test animal in and removes it from the apparatus. In addition, the experimenter generally scores the behavior of animals visually. "Blind" test procedures can provide only limited protection against possible bias because the very presence of the experimenter/observer alters normal open-field behavior in the rat (Hughes, 1978).

One solution for this problem is an automated open-field apparatus that provides very consistent scoring of behavior without experimenter intervention. Two general methods of activity monitoring are used in automated apparatus: (1) detection of position within the field by mechanical sensors, i.e., weight-activated floor panels; and (2) detection of motion by capacitive, radiofrequency (RF), or light sensors. Mechanical sensors provide a discrete measure of activity more comparable to observer scoring methods, but such apparatus were unacceptable for this project since they must be custom-built and do not have a proven record of reliability and repeatability of results. Commercially available motion-detection apparatus with capacitive or RF-field-disturbance technologies were also unacceptable because of possible interaction of exposure conditions with sensing fields.

An open-field apparatus with an infrared-light-emitting sensing schema was selected. This apparatus provided a readout of both activity/motion within the field and of position along the x-y axis of the field. The latter information was used to indicate an animal's field position in one

of the four possible quadrants. The data collected for each animal included a general activity count; i.e., beam interruptions, the number of quadrants entered, their spatial distribution, and the number of moves between quadrants. In addition, at the end of each test session the field was inspected for signs of urination and defecation.

All open-field assessments were made by means of two commercial open-field apparatus (Opto-Varimax-Magnus, Columbus Instruments, Columbus, Ohio). Each was a 100-cm-square corral consisting of four transparent Plexiglas walls 50 cm high, supported by an external aluminum framework at each corner with connecting cross supports along each side (Fig. 1). Each apparatus was held approximately 100 cm above the floor by a wooden tablelike frame that provided support at each corner and along the bottom edge of each wall. A 100-cm-square Plexiglas sheet was hinged to the underside of this frame on one side, with a latch on the opposite side, thus forming a "trapdoor" floor for the open field (Fig. 2). This floor arrangement facilitated uniform removal of the animals from the field and simplified the between-trials cleaning procedure. A fiberboard lid covering the top of the field contained the animal within the field during testing and blocked extra-field visual stimuli. The walls and the underside of the trapdoor were covered on the outside of the field with light-brown paper. These precautions resulted in uniform indirect illumination of the field.

Light intensity measured at the floor of the field with the lid open and closed indicated average illumination levels of approximately 10 lux and .25 lux respectively. The average sound level during testing was approximately 40 dBA (re 20 μN/m^2).

Arrays of infrared-light-emitting diodes (LED) and phototransistors were concealed within the metal framework on two adjacent sides to provide a crisscrossed pattern of light beams 5 cm above the floor of the field. The electrical signals from the phototransistors were monitored by an electronic control unit, and each interruption of an LED/phototransistor beam path by movement of an animal was tallied and displayed. A LED display also indicated the x-y position of the animal within the field (Fig. 1).
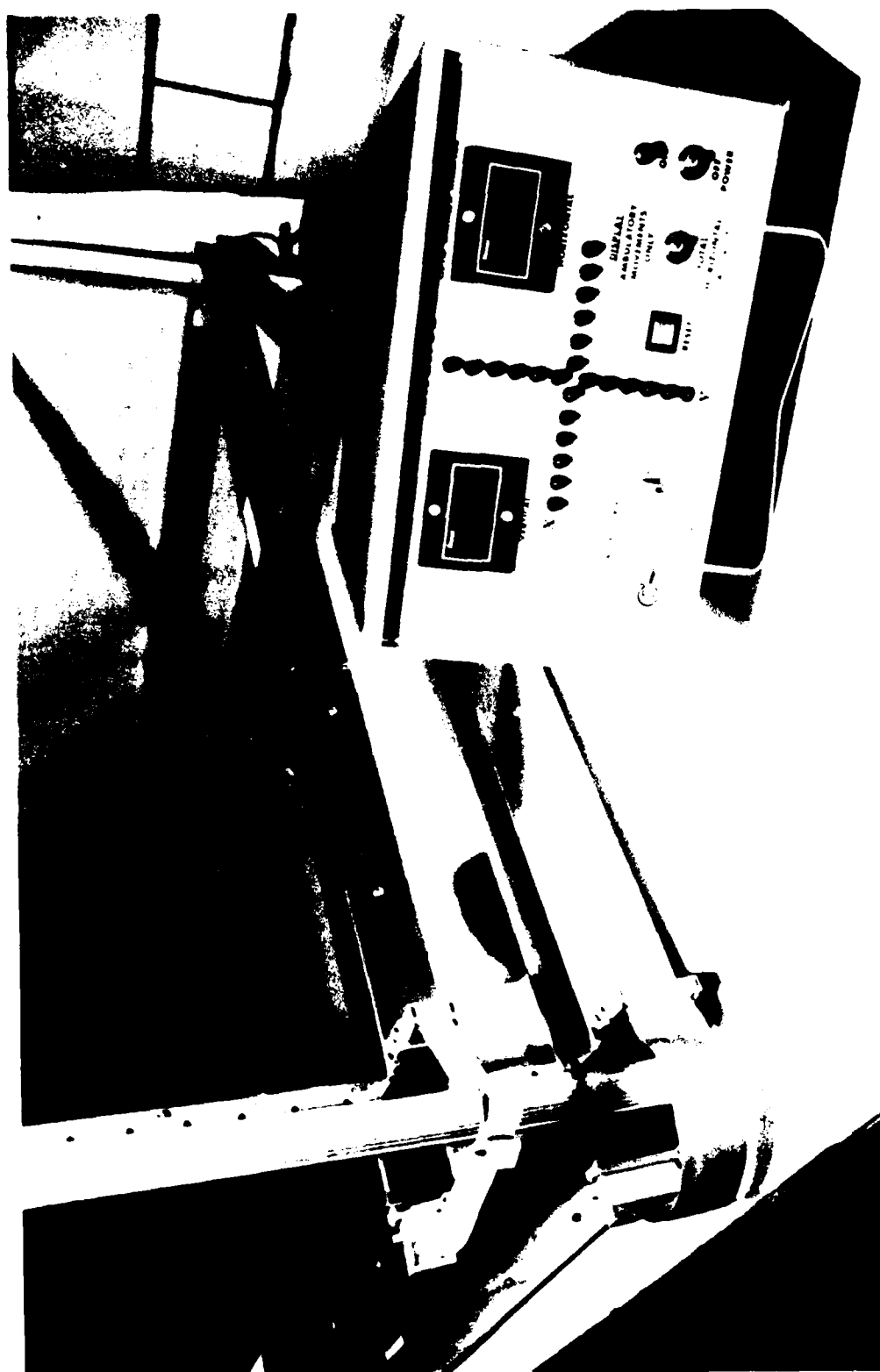
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Figure 1. Open-field apparatus and electronic control unit used during pilot testing of the device.

Figure 2. Technicians remove an animal from the underside of the field via a trapdoor floor during simulated assessment period.

Procedure

All exposed and sham-exposed animals were tested at regular intervals in the open field. So as to not to interfere with the routine assessment of blood chemistry, hematology, and corticosterone, we scheduled an open-field test 3 weeks prior to each bleeding session. Thus open-field testing was conducted once every 6 weeks during the first 15 months of the project and at 12-week intervals in the final 10 months. During the course of the project, 14 open-field assessments were made. These assessments were conducted outside the animal rooms, in the clean hallway of the SPF facility, during the 2-h period the animals were housed in holding bins as part of the waveguide-maintenance procedures.

To integrate the open-field assessment of all 200 animals into the daily SPF maintenance schedule, we tested 40 animals per day over a 5-day period. Two alcoves were randomly selected each day, and the 40 animals from these alcoves were randomly ordered for testing. We divided them between the two apparatus, balancing across treatment conditions throughout repeated testing.

Additional personnel were assigned to the clean hall to conduct the open-field assessments so as not to disrupt normal daily procedures within the exposure rooms. At approximately 5-min intervals the holding bin of a preselected animal was taken to the clean-hall doorway and passed across the threshold to a technician in the clean hall. This technician transported the bin to yet another technician, who operated the open-field apparatus. The technicians in the clean hall were unaware of the treatment condition (exposed or sham exposed) of the animal under test.

The animal was removed from its bin and lowered by the tail into the center of the field, and the lid was closed. After a single 3-min test period, the trapdoor floor was carefully lowered a few inches and the animal was retrieved from the field (Fig. 2). The animal was returned to its holding bin and transported back to the exposure room. While the trapdoor was in the down position, the floor of the field was wiped clean of urine and fecal matter prior to testing of the next animal. The data for each animal were recorded in a logbook and the instrumentation was reset.

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Results

The mean activity counts for exposed and sham-exposed animals throughout all 14 test sessions are presented in Fig. 3. The overall activity pattern is remarkably similar for both groups, except for an apparent difference between them during the first test session.

The activity counts for each of the 14 sessions are summarized in Table 1. The coefficient of skewness is zero for symmetrical distributions, positive for distributions skewed to the right, and negative for distributions skewed to the left. During the first open-field assessment, the distribution of activity counts for the sham-exposed animals is skewed to the right, so the mean count was inflated by a small number of relatively active animals. The distribution for the exposed animals is close to zero, indicating a reasonably symmetrical distribution of data. In each of the remaining 13 sessions the activity-count distributions for sham-exposed and exposed animals are similar, with positive coefficients of comparable magnitude. More specifically, the distributions are skewed to the right, so the estimated mean counts for the groups were inflated by a few active animals.

For higher levels of activity, the standard deviations are larger; so as activity levels increased, the range of activity was more varied. Moreover, the standard deviation (variance) is large for each session. While this was not a direct consequence of the 14 sessions, the pattern of activity of each animal showed very little consistency over the 14 sessions. We attempted to model the pattern of each animal over the sessions for which complete data were available; however, none of the models fitted the data well.

Since the standard deviation was dependent on the mean, the raw data were transformed prior to further analysis so as to reduce this dependence. Because the raw data represented "counts," the square-root transformation was used and found to be well suited. Various parameters for the transformed data are presented in Table 2. The coefficient of skewness for the data is now close to zero for both groups throughout all but the first session. In that session the distribution for the exposed animals is

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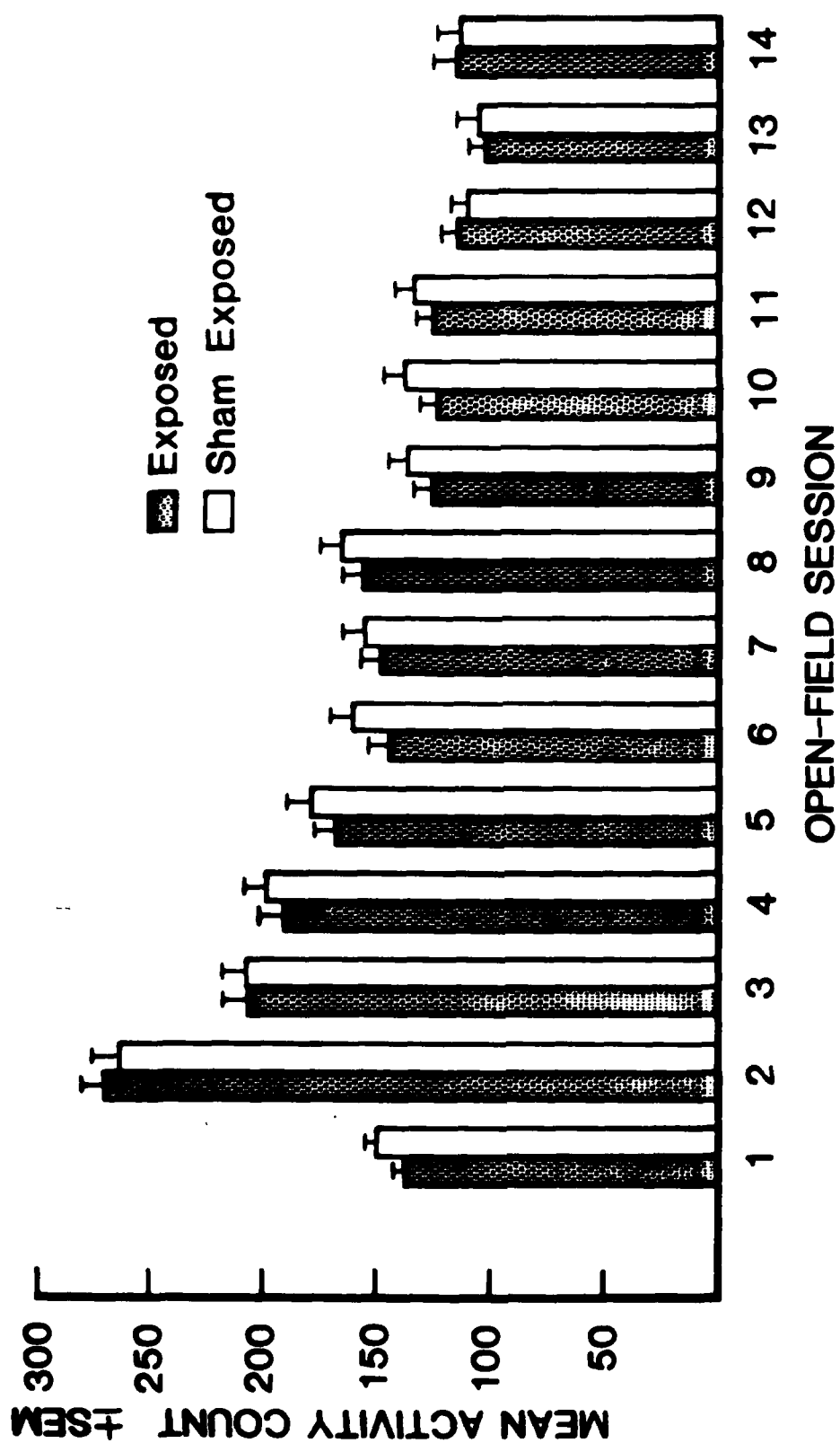
Figure 3. Comparison by treatment group of mean levels of activity throughout the 14 open-field assessment sessions.

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TABLE 1. RAW ACTIVITY COUNTS FOR EXPOSED AND SHAM-EXPOSED POPULATIONS

	Session	No. in Group	Median	Max	Min	Mean	S.D.	Skewness
EXPOSED	1	98	141	274	10	137.2	53.7	0.12
	2	97	259	650	75	268.9	124.0	0.41
	3	97	192	515	38	206.1	114.9	0.69
	4	97	169	577	22	189.8	118.1	0.76
	5	96	153	509	14	166.9	99.9	0.88
	6	95	125	464	15	144.4	94.8	0.69
	7	92	140	492	12	147.5	91.6	0.80
	8	91	136	457	28	155.2	95.8	0.90
	9	78	103	377	19	124.9	79.5	0.95
	10	76	119	327	26	122.6	65.7	0.71
	11	68	113	298	31	124.8	64.9	0.78
	12	60	99	326	15	115.4	68.1	1.19
	13	41	98	265	20	102.6	53.2	0.89
	14	26	98	254	38	114.8	49.2	0.82
SHAM	1	99	149	306	55	150.8	44.2	0.40
	2	97	258	646	55	262.1	130.0	0.54
	3	96	167	631	14	207.5	125.1	0.80
	4	94	182	582	11	197.6	113.9	0.92
	5	90	163	436	33	178.3	94.3	0.68
	6	89	143	443	2	160.1	99.4	0.57
	7	88	147	381	13	155.3	85.2	0.67
	8	87	157	450	22	165.3	93.9	0.79
	9	74	120	345	25	135.9	76.5	0.92
	10	70	137	344	20	138.3	76.1	0.68
	11	67	116	430	33	131.7	83.1	1.34
	12	60	102	245	15	110.8	58.9	0.50
	13	40	99	279	24	103.5	59.3	0.78
	14	22	102	213	50	112.9	51.1	0.61

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TABLE 2. TRANSFORMED ACTIVITY COUNTS FOR EXPOSED AND SHAM-EXPOSED
POPULATIONS

	Session	No. in Group	Median	Max	Min	Mean	S.D.	Skewness
EXPOSED	1	98	11.9	16.6	3.2	11.4	2.4	-0.59
	2	97	16.1	25.5	8.7	15.9	3.9	-0.06
	3	97	13.9	22.7	6.2	13.8	4.0	0.17
	4	97	13.0	24.2	4.7	13.1	4.3	0.13
	5	96	12.4	22.6	3.7	12.3	3.8	0.27
	6	95	11.2	21.5	3.9	11.3	4.0	0.10
	7	92	11.9	22.2	3.5	11.5	3.8	0.09
	8	91	11.7	21.4	5.3	11.9	3.8	0.29
	9	78	10.1	19.4	4.4	10.6	3.5	0.32
	10	76	10.9	18.1	5.1	10.8	3.0	0.18
	11	68	10.6	17.3	5.6	10.8	2.9	0.30
	12	60	10.0	18.1	3.9	10.3	3.0	0.48
	13	41	9.9	16.3	4.5	9.8	2.6	0.32
	14	26	9.9	15.9	6.2	10.5	2.2	0.33
SHAM	1	99	12.2	17.5	7.4	12.1	1.8	-0.10
	2	97	16.1	25.4	7.4	15.7	4.1	0.08
	3	96	12.9	25.1	3.7	13.7	4.4	0.15
	4	94	13.5	24.1	3.3	13.4	4.2	-0.03
	5	90	12.8	20.9	5.7	12.9	3.6	0.07
	6	89	12.0	21.0	1.4	12.0	4.1	-0.09
	7	88	12.1	19.5	3.6	12.0	3.5	0.00
	8	87	12.5	21.2	4.5	12.3	3.7	0.13
	9	74	11.0	18.6	5.0	11.2	3.2	0.31
	10	70	11.7	18.5	0.0	11.2	3.5	-0.34
	11	67	10.8	20.7	5.7	11.0	3.4	0.56
	12	60	10.1	15.7	3.9	10.1	2.8	0.08
	13	40	10.0	16.7	4.9	9.8	2.9	0.27
	14	22	10.1	14.6	7.1	10.4	2.3	0.38

negative, i.e., the mean count was deflated by a number of relatively inactive animals. Also as a result of the transformation, the standard deviation does not appear to depend on the session number. In addition, comparison of "normal probability plots" for the original and transformed data indicated that the transformed data are more consistent with the assumptions of normality inherent in the statistical procedures applied and discussed below.

The correlation matrices for both groups are given in Table 3. As can be seen, there are no negative correlations; thus, there were few reversals in behavioral patterns throughout the course of the 14 assessment periods. As expected, the strength of correlation between the mean activity levels for two sessions depends on the "time distance" between the sessions. With the exception of the first two sessions and the last two sessions, correlations between consecutive sessions are strong. However, inspection of bivariate plots suggests that the standard errors for these correlations are large, so the correlations may not be well determined.

Since complete records were available only for the animals that survived to participate in all 14 test sessions, the usual multivariate procedures used for analysis of repeated measures could not be used to evaluate the data from all sessions. Inherent in these methods is the assumption that "missing data" are missing owing to random processes and may therefore be replaced prior to analysis by an estimate calculated from the available data. This assumption is not valid for this set of data since a systematic process, mortality, is responsible for the end of most records being incomplete.

Therefore, an initial analysis with the t-test was performed on the raw and transformed data from all test sessions. A summary of this analysis is presented in Table 4. The p-values across sessions apart from session 1 provide no evidence of differentiation between the groups. In session 1 the exposed-animal level of activity appears to be lower (approximately 9%) than that of the sham-exposed animals.

Prior to the start of the experiment we suspected that the initial open-field session would be the most informative in a series of repeated assessments, so the data for session 1 is evaluated separately. On the basis of the t-test there is evidence that activity level was significantly lower for the exposed population than for the sham-exposed population (t =

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TABLE 3. CORRELATION MATRICES FOR OPEN-FIELD ACTIVITY TEST SESSIONS

Session	1	2	3	4	5	6	7	8	9	10	11	12	13	14
EXPOSED 1	1													
2	.45	1												
3	.31	.65	1											
4	.37	.58	.71	1										
5	.31	.41	.52	.66	1									
6	.28	.48	.44	.56	.64	1								
7	.30	.44	.49	.59	.60	.73	1							
8	.23	.41	.51	.58	.57	.57	.76	1						
9	.23	.38	.42	.43	.45	.47	.63	.66	1					
10	.26	.42	.34	.39	.45	.46	.66	.67	.71	1				
11	.23	.25	.15	.30	.36	.44	.66	.59	.58	.72	1			
12	.22	.18	.14	.15	.18	.24	.51	.46	.45	.52	.55	1		
13	.04	.18	.15	.29	.16	.35	.47	.46	.44	.46	.42	.43	1	
14	.09	.02	.07	.14	.12	.14	.15	.29	.27	.21	.26	.31	.32	1
SHAM 1	1													
2	.49	1												
3	.48	.76	1											
4	.36	.51	.68	1										
5	.20	.47	.59	.63	1									
6	.21	.35	.46	.51	.59	1								
7	.16	.25	.16	.27	.46	.59	1							
8	.19	.30	.26	.30	.48	.58	.73	1						
9	.16	.28	.29	.28	.30	.38	.50	.58	1					
10	.19	.16	.16	.23	.30	.33	.42	.52	.68	1				
11	.07	.08	.08	.15	.20	.28	.39	.42	.39	.51	1			
12	.20	.18	.20	.20	.24	.38	.47	.45	.42	.45	.56	1		
13	.10	.13	.05	.07	.22	.26	.43	.48	.43	.53	.51	.67	1	
14	.06	.16	.00	.00	.06	.03	.18	.22	.21	.18	.18	.11	.16	1

TABLE 4. RESULTS OF t-TESTS OF RAW AND TRANSFORMED ACTIVITY COUNTS

	Raw Data			Transformed Data		
Session	t	p	df	t	p	df
1	-1.94	.053	195	-2.24	.026	195
2	0.37	.709	192	0.46	.649	192
3	-0.08	.939	191	0.10	.923	191
4	-0.47	.641	189	-0.58	.564	189
5	-0.80	.426	184	-0.96	.337	184
6	-1.09	.277	182	-1.06	.291	182
7	-0.59	.557	178	-0.80	.425	178
8	-0.71	.479	176	-0.81	.418	176
9	-0.87	.385	150	-1.08	.281	150
10	-1.34	.182	144	-1.08	.280	144
11	-0.54	.593	133	-0.29	.774	133
12	0.39	.694	118	0.32	.750	118
13	-0.07	.942	79	0.06	.950	79
14	0.13	.897	46	0.17	.867	46

-2.24, p = .026, df = 195). The 9% reduction in activity represents approximately 30% of the standard deviation associated with either treatment condition during the first test session.

A more appropriate treatment of the activity counts requires a multivariate analysis that takes into account the correlations between sessions. The records were examined to obtain as complete a set of data as possible for such an analysis; during the first eight sessions complete records were available for 88% of the animals. The analysis was then based on this truncated set of data, and a Hotelling T-square statistic of 8.73, p = .40, with (8,168) degrees of freedom (df), was obtained. Therefore, when each of the first eight sessions is given equal weight, no longitudinal differences are indicated between the two treatment groups.

This latter conclusion does not contradict the finding of the t-test of mean activity counts during the first session. It does indicate that the significant difference between treatment groups in their reaction to the open field during the first assessment period was not very large, and as expected, not generalized within the context of the first eight sessions. Analysis of complete records available for sessions 5 through 12 provided similar results.

Further examination of the mean activity counts (presented in Fig. 3), suggests that the level of activity varied as a function of the session number, decreasing monotonically after the second session. The session number reflects the number of previous exposures to the test apparatus and also the age of the animals. On the basis of complete records available for the first eight sessions, we conducted an analysis to determine whether the mean activity count varied with session number (Timm, 1971).

Formally, let u_{E1} = mean count for the exposed animals tested in session 1 and let u_{S1} be similarly defined for the sham-exposed animals. We wish to test the hypothesis that

$$u_{E1} = u_{E2} = u_{E3} \cdots = u_{E8}$$
$$u_{S1} = u_{S2} = u_{S3} \cdots = u_{S8}$$

Treating both groups separately, we find that the value for the Hotelling T^2 statistic for the exposed animals is 34.05 ($p < .001$, df = 7,90). Hence there is strong evidence that the mean level of activity is dependent on the session number. Simultaneous confidence intervals were computed to locate the differences that might have led to the rejection of the hypothesis. The analysis indicated that the activity levels for session 1 and sessions 5-8 were equivalent, with higher levels of activity in sessions 2-4. Formally, the analysis showed that

$$u_{E1} = u_{E5} = u_{E6} = u_{E7} = u_{E8}$$
and $\quad u_{E2} \neq u_{E3} \neq u_{E4}$

A similar result was obtained in the analysis for the sham-exposed animals, a Hotelling T^2 statistic of 36.43 ($p < .001$, df = 7,88). Sessions 9-14 were not included in these analyses, but examination of their means suggests that the level of activity in these sessions was possibly lower.

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The quadrant-entry data were organized as a set of tables, and an ordered categorical analysis of the relationship between the number of quadrants entered and the treatment condition was completed with the Wilcoxon test. No such association was seen.

Results of the activity-data evaluation in terms of number of moves between quadrants, i.e., quadrant changes, are presented in Fig. 4. The pattern of response throughout the 14 test sessions is quite similar to that observed for general activity, discussed above. The estimated standard deviations for both exposed and sham-exposed treatment groups are comparable and stable throughout all sessions. As the distributions for both groups are skewed only slightly to the right, analysis was completed using the raw data without transformation. This analysis gave no evidence of any differences in movement between quadrants.

The frequency of urination during open-field testing is presented in Fig. 5. On the basis of the Wilcoxon test, no association was seen between frequency of urination and treatment condition. The frequency of defecation is presented in Fig. 6. The data were similarly analyzed, and again there was no evidence of any association between defecation and treatment condition.
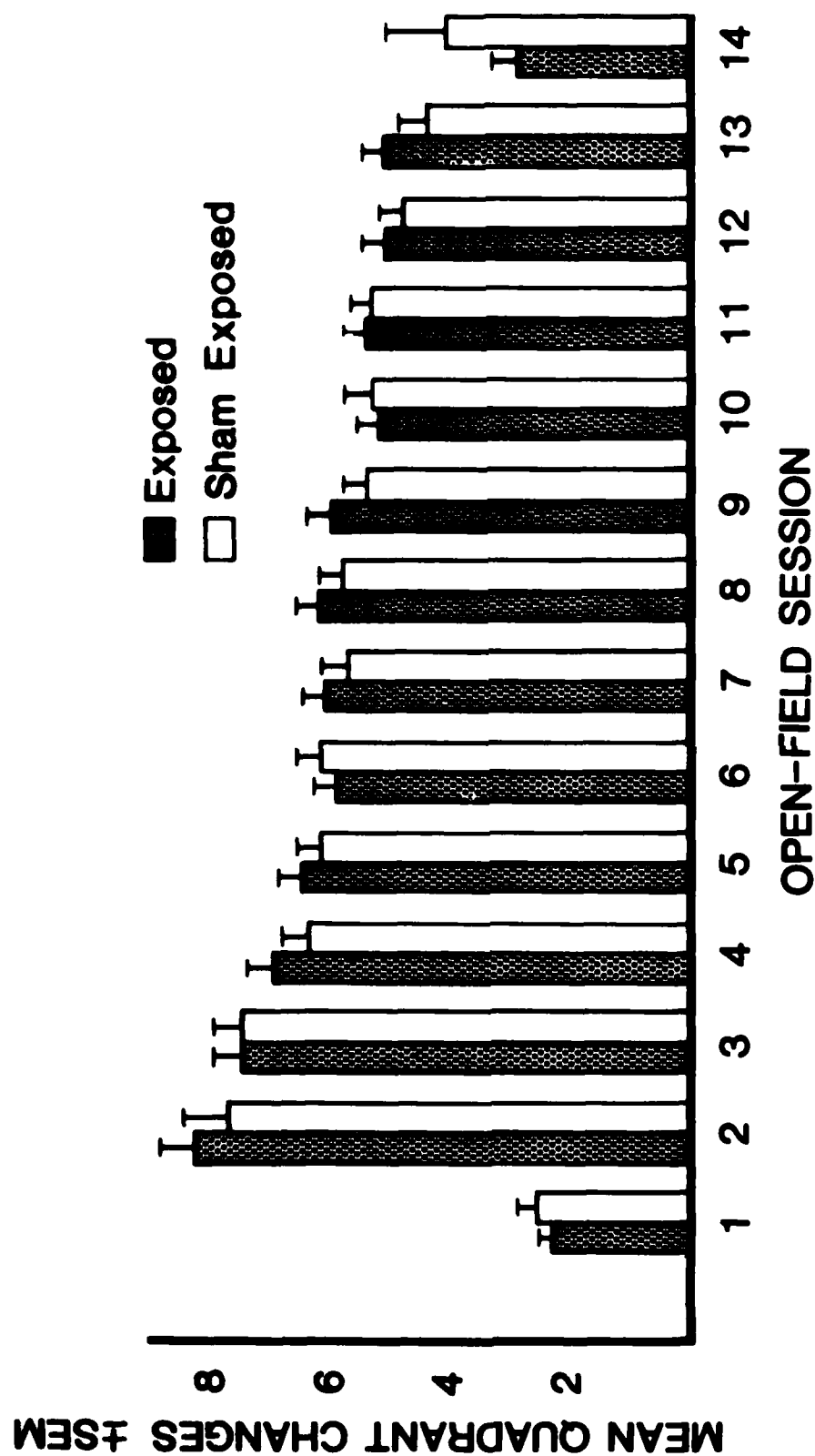
Figure 4. Comparison by treatment group of mean number of moves between quadrants in 14 sessions of open-field testing.
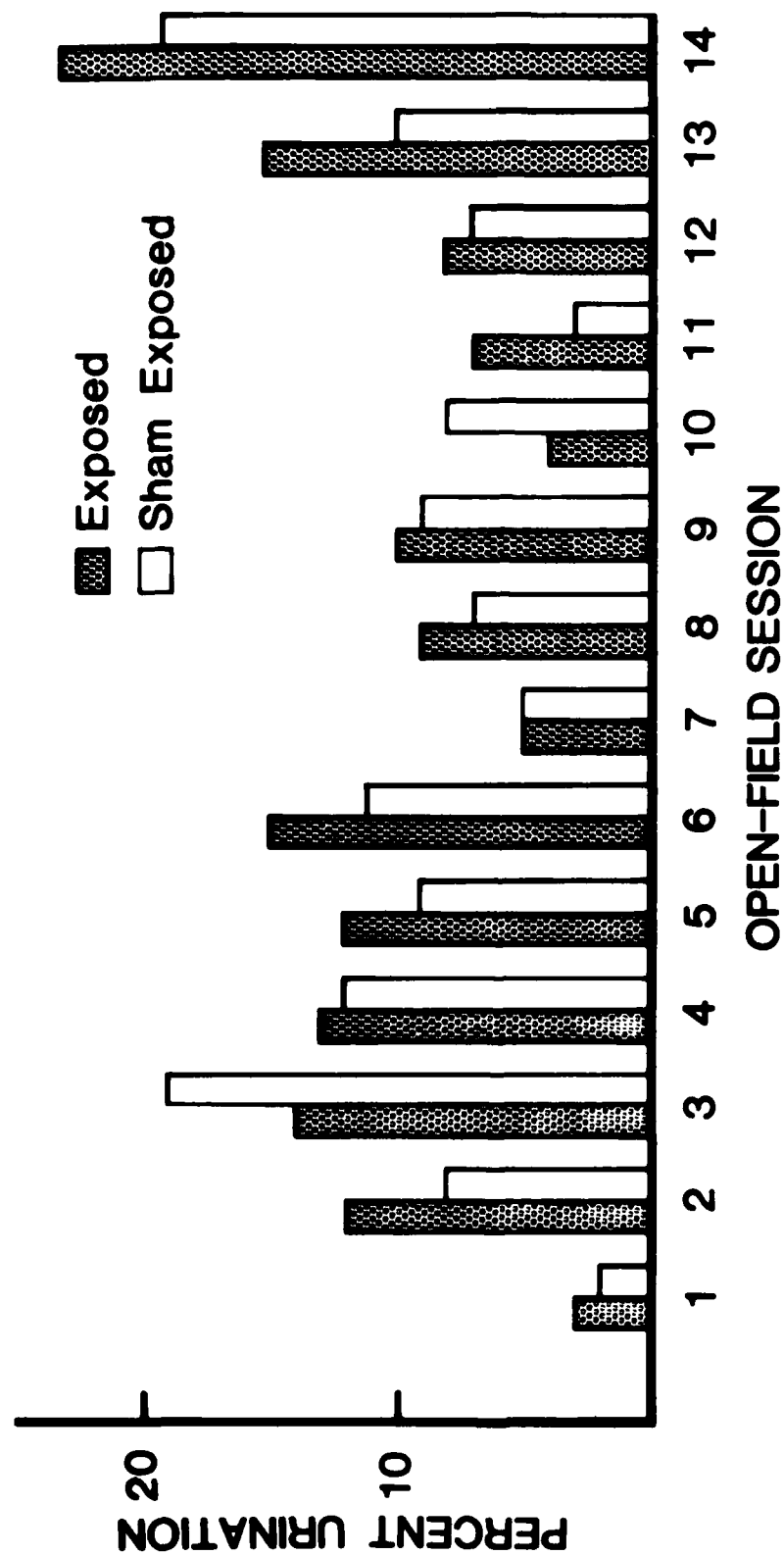
20

Figure 5. Comparison by treatment group of percentages of animals urinating in 14 sessions of open-field testing.
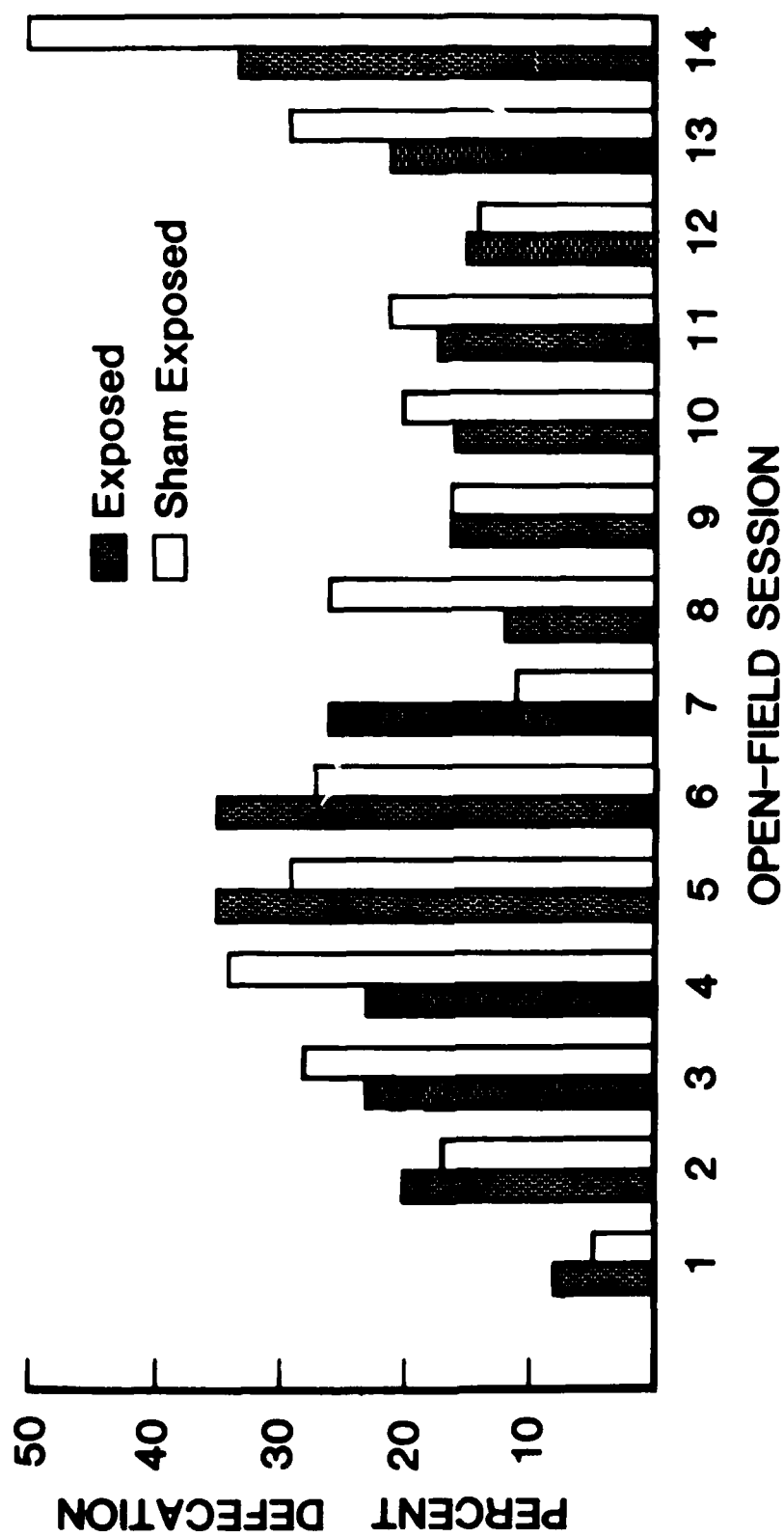
Figure 6. Comparison by treatment group of percentages of animals defecating in 14 sessions of open-field testing.

Discussion

Analysis of the data from the 14 open-field assessments supports the conclusion that, except for the first test session, 2 years of exposure to low-level pulsed-microwave radiation did not lead to significant alterations in behavior as measured by activity, defecation, or urination. During the first test session, the general activity level of the exposed animals was significantly lower, by approximately 9%, than that of the sham-exposed animals.

The open-field test has been most commonly applied as a measure of emotionality, with reduced activity and increased defecation or urination regarded as indicative of heightened emotionality, fear, or stress (Archer, 1973; Walsh and Cummins, 1976). Although the validity of the activity measure as an emotionality index has not gone unchallenged, there is general agreement that defecation and possibly urination are valid indices (Hall, 1934, 1936; Pare, 1964a; Whimbey and Denenberg, 1967; Denenberg, 1969; Ivinskis, 1968, 1970; Seliger, 1978; Tachibana, 1980, 1982).

Reduced activity in the first session is not supported by the increased defecation expected with increased emotionality or stress. The normal usefulness of the defecation/urination measures may have been voided by the animals being handled daily during their transfer from waveguide to holding bin. It has been reported that handling or "gentling" a rat reduces emotionality and leads to increased activity and decreased defecation in the open-field test and a reduction of the validity of the defecation measure (Doty and Doty, 1967).

Apart from the initial difference between the treatment groups during session 1, their patterns of general activity throughout the 14 test sessions were strikingly similar. Behavior increased from the start, attained its highest level during session 2, and gradually declined throughout the remaining sessions.

It has been reported that rats display an age-related increase in open-field behavior through the first 5-6 months of age, followed by an age-related decrease (Broadhurst, 1957; Furchtgott et al., 1961; Doty and Doty, 1967). Similar reports indicate an apparent age-dependent increase and subsequent reduction of activity-wheel behavior (Munn, 1950; Desroches et al., 1964) and general locomotor activity (Jones et al., 1953;

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Montgomery, 1955; Hofecker et al., 1974). Other reports indicate that repeated testing in an open-field apparatus leads to a gradual reduction in activity levels, due to processes related to habituation or reduction of emotionality, and that this pattern may be preceded by an initial inhibition of behavior followed by a marked increase (Hall, 1934; Broadhurst and Eysenck, 1964; Pare, 1964b; Whimbey and Denenberg, 1967; Valle, 1971; Russell and Williams, 1973).

Therefore, it appears that the open-field activity pattern during the course of this study resembles that normally observed as a function of age/experience and was not affected by a lifetime of low-level pulsed-microwave exposure.

CORTICOSTERONE

As a result of physical or psychological trauma, whether real or perceived, the pituitary gland releases the adrenocorticotropic hormone (ACTH) into the circulatory system. When ACTH reaches the adrenals of the rat, it stimulates them to release corticosterone, an endocrine hormone, which functions as a mobilizing agent for various physiological processes required to cope with sustained stress situations.

During the past 30 years circulating levels of corticosterone have been utilized as an indirect measure of the stress associated with a general experimental environment or a specific treatment condition. Circulating levels of corticosterone in rats vary, as reported in the literature, from as low as 2-3 μg/100 ml to as high as 80 μg/100 ml. Many factors, not specifically stressful, influence the circulating levels of corticosterone in rats. Generally these levels follow a sinusoidal pattern throughout each circadian period, with the trough occurring normally in the early morning hours and the peak about 12 h later (Guillemin et al., 1959; Saba et al., 1963; Dunn and Scheving, 1971; Dunn et al., 1972; Seggie et al., 1974). This pattern can be reversed if the animal is maintained on a reversed day/night cycle, with room lighting "off" during the daytime hours and "on" during the nighttime (Scheving and Pauly, 1966). The mean value of this sinusoidal pattern will vary in response to other known factors. For example, females invariably have higher corticosterone levels than males (Kitay, 1963; Critchlow et al., 1963; Tachi et al., 1981), and prolonged individual housing leads to higher levels than group housing (Stern et al., 1960; Hatch et al., 1965; Plaut and Grota, 1971; Lovely et al., 1972).

Superimposed on this circadian background, specific effects of acute treatments can readily be discerned. Removing a rat from its cage causes a marked rise in serum corticosterone beginning approximately 3-4 min later. Even if the stimulus of handling is removed immediately, corticosterone levels will continue to rise and will require 30-60 min to return to baseline levels (Barrett and Stockham, 1963; Ader and Friedman, 1968; Seggie and Brown, 1975). The magnitude of the corticosterone elevation

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associated with a stimulus has been determined to be a graded function of stimulus intensity and duration (Ader and Friedman, 1968; Hennessey et al., 1979; File and Peet, 1980; File, 1982). In addition, the magnitude of this response is itself a function of the circadian pattern and other environmental determinants (Zimmerman and Critchlow, 1967; Plaut and Grota, 1971; Dunn et al., 1972; Seggie and Brown, 1975).

We periodically assessed corticosterone as part of the protocol for this long-term project for two purposes: (1) to monitor the general environment of the experimental animals and the handling procedures required by the daily maintenance schedule; and (2) to test specifically for corticosterone changes resulting from microwave exposure.

During the first year of the project five corticosterone sampling sessions were completed, at 12-week intervals, coincident with every other regularly scheduled bleeding session. During the second year, corticosterone samples were taken only 6 weeks before and at the time that the last surviving animals in each group were killed.

Materials and Methods

Blood Sampling

Although the reactivity of endogenous levels of corticosterone makes it a sensitive endpoint for studying biological effects of environmental stimuli, its naturally occurring variability has led to some standardization of sampling methods in an attempt to achieve comparability between investigators. Specifically, blood samples drawn for corticosterone must be obtained within 2-3 min after removal of a rat from its cage (cf. Zimmerman and Critchlow, 1967; Davidson et al., 1968). Obtaining blood samples within this time limit ensures that the assayed values reflect the circulating levels of corticosterone maintained in response to the general environment of the animal or the specific experimental treatment condition and not simply the transitory excitatory response due to the immediate handling of the animal.

The blood-sampling procedure is described in detail in Volume 6 of this series; the following is a brief summary.

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The blood sampling procedure was in part determined by the daily caretaking routine of the SPF facility and microwave exposure apparatus. The animals were removed daily from their waveguides, in staggered groups of 20 animals each, and housed temporarily in individual holding cages for approximately 2 h. After the waveguide cages had been cleaned, the animals were returned to them. During the 2-h holding period, the animals remained undisturbed for at least 30 minutes before some were randomly selected from that group for bloodletting. Each selected animal was then removed from its holding cage and anesthetized with a mixture of halothane, nitrous oxide, and oxygen. A single blood sample was taken for both the regular blood chemistry and the corticosterone determinations, using the retro-orbital technique. For the various blood chemistry and corticosterone assays to be performed on the sample, 2 ml of whole blood was needed. With few exceptions all samples were obtained within 2 min of disturbance of an animal's holding bin. So that the sampling of blood would fall within the trough of the circadian fluctuation of corticosterone, only 50 animals were bled daily. Half of these were exposed animals and half were sham exposed. This schedule required a 4-day period for procurement of samples from all 200 animals. The portion of the blood drawn for corticosterone assay was prepared and frozen for later laboratory analysis.

These procedures were followed on six of the seven corticosterone-sampling periods completed. The seventh sampling period was coincident with the kill of the surviving animals at the termination of the project. The procedure was different in that after removal from the waveguide cages, the animals were weighed, placed in their holding bins, and then placed on a laboratory cart. Once all animals were on the cart it was wheeled outside the necropsy room door. The exposed and sham-exposed animals were then taken in random order, individually, into the necropsy room over a 30-min period and were anesthetized and exsanguinated. The normal undisturbed time prior to blood sampling did not apply here.

To isolate the run-to-run variation traceable to minor procedural variations in the assay technique used, we prepared a serum pool prior to the start of the study. One hundred animals that had participated in pilot studies during early phases of the project were anesthetized and exsanguinated via decapitation or cardiac puncture. The serum was pooled

and frozen. At each sampling session throughout the study, 20 aliquots of this serum were randomly interspersed with the actual samples sent to the laboratory for corticosterone analysis. All the samples were labeled with a code so that the assays were run under "blind" conditions.

Assay

The microfluorimetric assay is based on a procedure described by Glick et al. (1964). The microassay employed was designed for use with 50-ul plasma or serum samples (Riley and Spackman, 1977; Spackman et al., 1978) and gives accurate and dependable results for quantities of corticosterone ranging from 0.1 to 50 ng (2 to 1000 ng/ml).

Preparation of glassware is a critical step in any fluorimetric procedure and particularly so in a microassay. The procedures chosen, after extensive evaluation, provided thorough cleaning, minimized contamination interference, and enabled a reasonably rapid routine with 12 or more assays per batch. All glassware was first rinsed six times or soaked in Liquinox cleaning solution, then rinsed in tapwater six times, in deionized distilled water three times, and in distilled acetone (from a polyethylene wash bottle) three times. The glassware was air dried for no more than 20 min, then covered with Saran film or stored in covered containers to prevent contamination from airborne particles and vapors.

Since disposable Pasteur pipettes and glass culture-tube cuvettes must be prewashed before use, they were routinely cleaned and reused repeatedly. Nonheparinized bleeding tubes were washed on a neoprene adapter in lots of 24 and then treated with sodium heparin.

All organic solvents--acetone, absolute ethanol, chloroform, methylene chloride, and carbon tetrachloride--were purchased as reagent grade and were distilled before use. Inorganic chemicals were reagent grade. Water was distilled and then passed through a deionizing purifying column.

The acid-alcohol reagent used to develop fluorescence was a critical component of the overall procedure. A two-to-one sulfuric acid/ethanol ratio gave maximum fluorescence with corticosterone and minimum fluorescence with interfering substances. The reagent was mixed in a glass-stoppered Erlenmeyer flask partially immersed in a crushed-ice bath.

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To provide low background blanks, the reagent was kept cold during both mixing and subsequent storage and was prepared fresh daily.

Samples of 50 μl of serum or working standards of corticosterone were pipetted into reaction flasks, then 650 μl of chloroform was added to each flask from a repetitive pipette dispenser. The flask was stoppered and the contents were agitated on a vortex mixer (in groups of four) for 30 s to extract the corticosterone. The flask was centrifuged (1 min), the aqueous layer was removed by controlled vacuum aspiration with a glass capillary tube, and 50 μl of 0.1 N NaOH was added. The flask was vortexed for 15 s and then centrifuged, and the aqueous layer was again removed. A 500-μl aliquot of the chloroform solution was transferred to another reaction flask containing 400 μl of the acid-alcohol reagent (pipetted before the extraction procedure was started). After 30 s of vortex mixing, followed by centrifugation, the upper chloroform layer was removed by aspiration and the total acid-alcohol volume was transferred to a 6- x 50-mm Pyrex culture-tube cuvette.

The fluorescence was allowed to develop in an incubator at 27°C. Readings were taken at 60 min and again at 70 min, starting from the time that the chloroform solution was mixed with the acid-alcohol reagent. A Turner model 430 spectrofluorometer was used to determine the fluorescence, with the excitation wavelength at 470 nm and the emission in wavelength at 525 nm. Fluorescence readings, properly corrected for the blank values, are linear with corticosterone concentrations when samples varying from 50 to 800 ng/ml are assayed. In using the model 430 spectrofluorometer, we adjusted the selected standard (200 ng/ml) to read 200 on the readout meter. The fluorescence in all other unknown samples was then read directly in ng/ml. To maintain the accuracy of assay-unknown samples, we had two or more standards in each batch of assays.

The small amounts of nonspecific fluorogens (NSF) present in physio-logical fluids were determined by using chromatography to separate the components on a silica-gel column. The total of NSF compounds was stable and reproducible in samples with different corticosterone concentrations. For each group of 200 serum samples, 20 to 30 samples with apparent low corticosterone levels were analyzed for NSF compounds. The average of the NSF compound totals from these selected samples was used as a constant, and all serum samples in the group were corrected for the true corticosterone

concentration by subtracting the NSF value from the apparent corticosterone concentration. Since seasonal or other variables may have had an effect on the NSF value, the NSF constant for each group (approximately 200 serum samples) was determined at each of the five sampling periods throughout the first year. The NSF value was fairly constant at 18 to 23 ng/ml.

Although we made all determinations and analyses using the full ng/ml resolution, we use the clinical terminology in the literature, i.e., $\mu g/100$ ml, to present all data so that these values can be more easily compared with those reported in the literature.

Results

A comparison of the control serum pool as assayed for each of the five sampling periods during the first year of the project and for the final two sampling periods approximately 1 year later is presented in Fig 7. Both the within-run and between-run variations are very small, indicating that the laboratory procedures and assay used produced highly consistent results. Since this run-to-run variation does not differentially affect the treatment means, the corticosterone data were not normalized prior to analysis.

During preliminary inspection of the data we observed that the variance of the data was uniformly high regardless of treatment condition and that the apparent baseline corticosterone level (indicated by the corticosterone levels of the sham-exposed animals) was not as low as can be obtained from nonstressed animals. These are not unexpected results since the daily maintenance procedures did not necessarily provide sufficient time for corticosterone levels of all animals to return to baseline after the temporary rise accompanying the initial removal of each animal from its waveguide. A number of "outlying" values ranged as high as 614 ng/ml. Since no criteria were available for selecting the data from animals that had not yet recovered from handling except the data themselves, all sampling data were included in subsequent analysis. Mean corticosterone levels from the five determinations for all animals during the first year of the project are compared in Fig. 8. These five periods correspond to bleeding sessions numbered 2, 4, 6, 8, and 10 in Volume 6 of this series of technical reports and to open-field sessions numbered 1, 3, 5, 7, and 9.
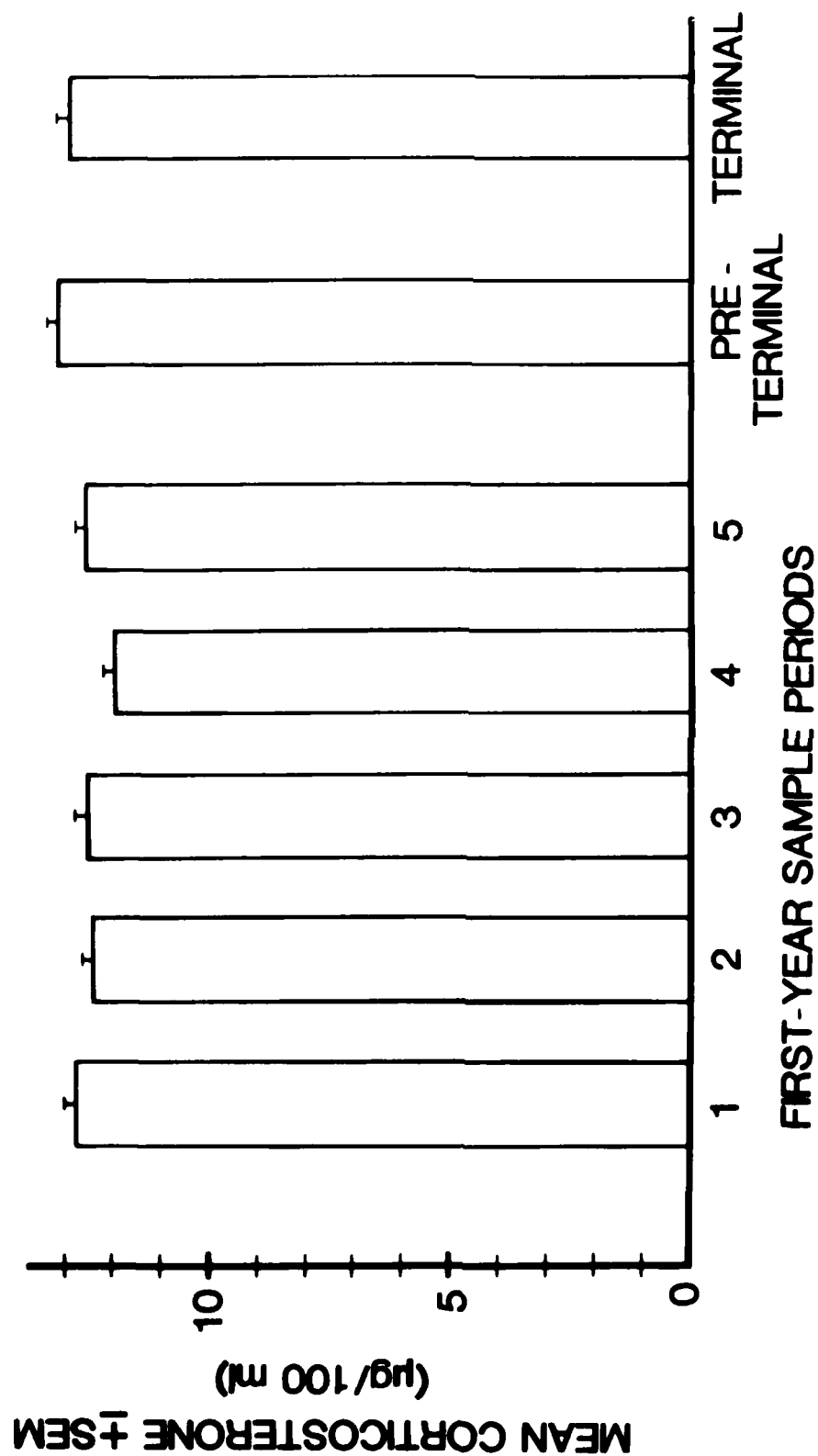
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Figure 7. Comparison of control corticosterone samples at each of the first five quarterly determinations and at the preterminal and terminal determinations.
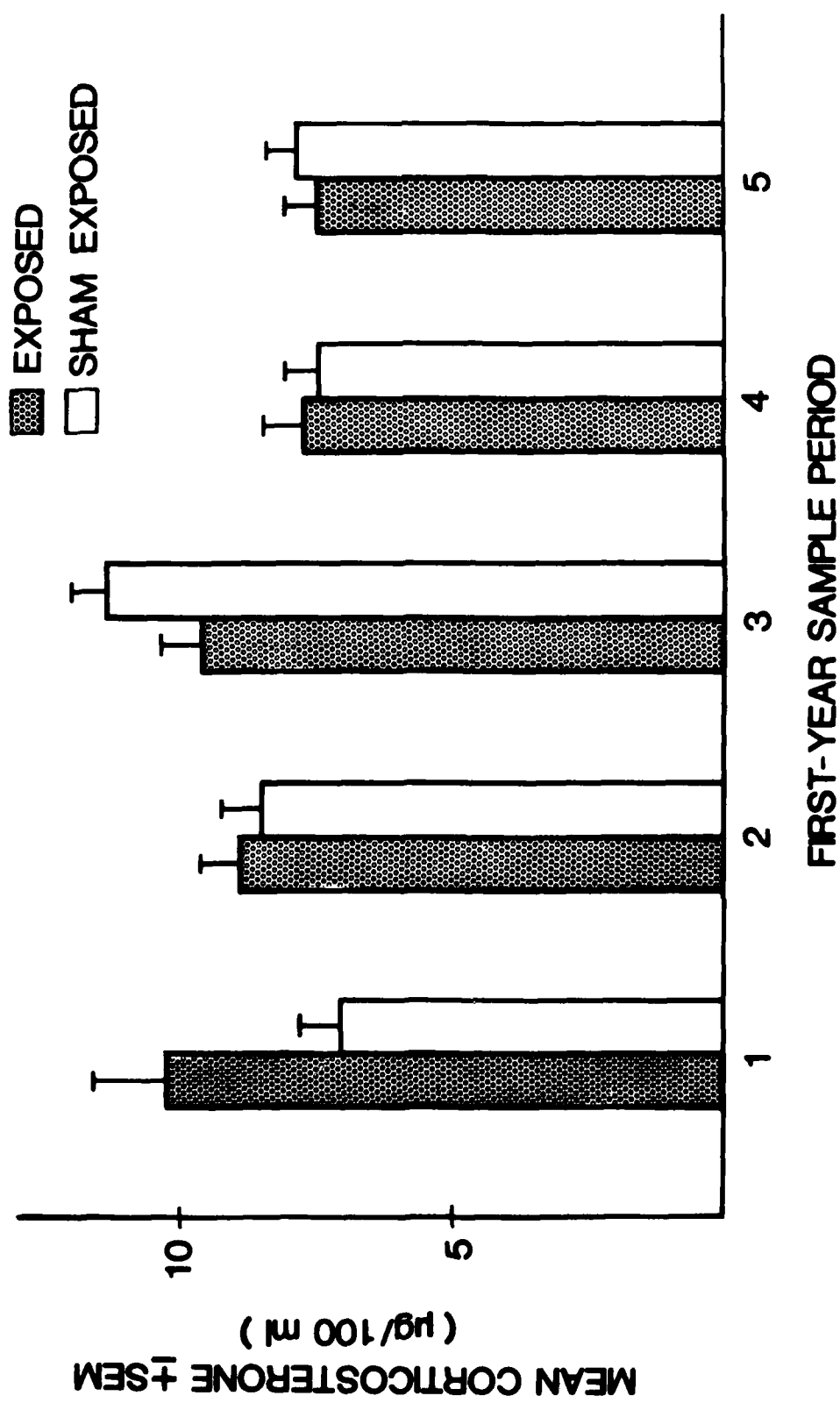
Figure 8. Comparison of mean corticosterone levels from five quarterly determinations during the first year of the project.

Inspection suggests that the exposed animals had a higher corticosterone level during the first sampling period than did the sham-exposed animals. During subsequent periods the exposed animals' corticosterone levels appear to have gradually decreased; the levels for sham-exposed animals, however, appear to have increased, and by the third period they exceeded the levels of the exposed animals. Corticosterone levels for the exposed and sham-exposed animals were apparently comparable during the fourth and fifth sampling periods.

A t-test of the data for the first five sampling periods indicated that the apparent difference between treatment groups during the first session was significant (t = 2.06, p = .04, df = 154), but that no differences were significant in the remaining periods. A multivariate analysis of all five periods, with the Hotelling T^2 statistic, indicated no overall differentiation of the treatment groups (F = 1.38, p =.24, df = 5,133).

Since the variance for all sample periods was high and the scatter plots of the data revealed the distributions to be highly skewed, a log transformation was completed on all data and additional analysis was performed.

A t-test of the transformed data indicated that the first period difference was marginally significant (t = 1.96, p = .052, df = 154) and the apparent third-period difference was significant (t = -2.25, p = .026, df = 161). At no other assessment period were corticosterone levels significantly different. A multivariate analysis of the transformed data indicated that, when equally weighted, the overall differences between the treatment groups were not significant (F = 2.09, p = .071, df = 5,133).

Correlation matrices for the raw data for both the exposed and sham-exposed treatment groups are presented in Table 5. The correlations between sessions are uniformly low regardless of treatment.

A problem associated with the multivariate analysis of this data is the abundance of missing data points. During any one period, 10-15% of the data for each treatment condition is missing because of failure to obtain the requisite blood volume. Only 40 animals from each treatment condition provided data at each of the five sampling periods. An analysis of both the raw and log-transformed data from these 80 animals showed differences to be nonsignificant in all pairwise comparisons as well as the multivariate analysis.

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TABLE 5.   CORRELATION MATRICES FOR RAW CORTICOSTERONE DATA FOR EXPOSED
           AND SHAM-EXPOSED TREATMENT GROUPS

Group		Session				
		1	2	3	4	5
	1	1.00				
	2	0.07	1.00			
EXPOSED	3	0.09	0.12	1.00		
	4	-0.00	0.05	-0.15	1.00	
	5	0.02	-0.03	0.06	0.20	1.00
	1	1.00				
	2	0.05	1.00			
SHAM	3	-0.06	0.17	1.00		
	4	-0.09	0.10	0.09	1.00	
	5	0.05	0.02	-0.09	0.02	1.00

The data on corticosterone from the final two sampling periods and the mean differences between these periods for both treatment groups are presented in Fig. 9.   These sampling periods correspond to bleeding sessions numbered 14 and 15 in Volume 6.   The preterminal corticosterone levels appear comparable, but the terminal measure for the exposed group is approximately 20% below that for the sham-exposed animals.   In addition, the terminal corticosterone levels for both groups appear elevated compared with the preterminal levels.   This latter difference suggests that the handling procedures accompanying the terminal collection were mildly stressful to both exposed and sham-exposed animals; thus, this difference can be considered as an indicator of the adrenocortical system's responsiveness to this novel situation.   If so, the adrenocortical responsiveness is approximately 60% less in exposed animals than in the sham exposed.

A t-test of both the raw and log-transformed data for the preterminal measure indicated no difference between the treatment groups. A comparison of the preterminal data (Fig. 9) with first-year data (Fig. 8) indicates that the corticosterone levels at the preterminal period were comparable to
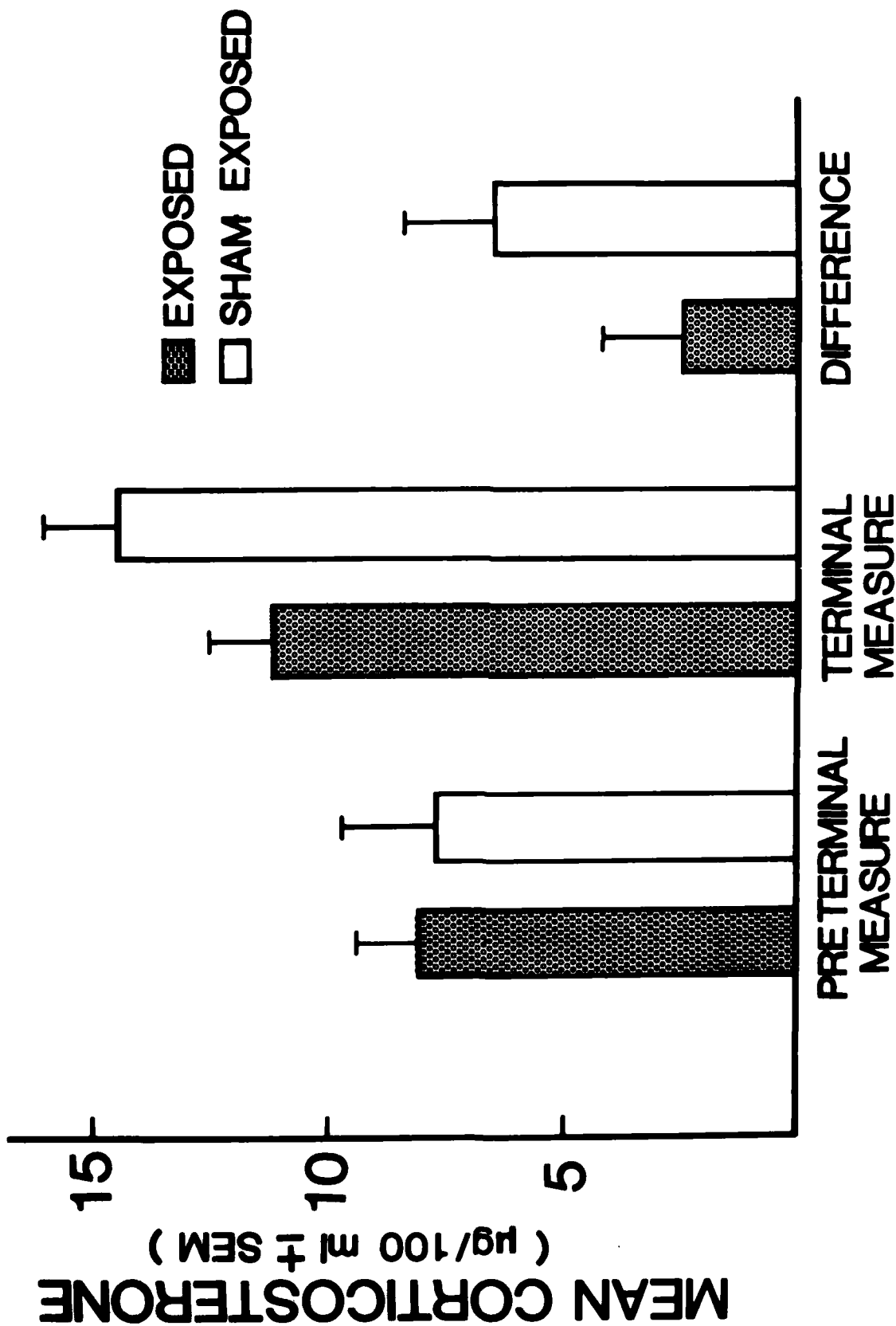
Figure 9. Comparison of mean corticosterone levels for animals represented in the terminal determination coincident with the final kill and those in a preterminal determination made 6 weeks earlier.

those during the fourth and fifth sampling periods of the previous year. The overall elevation of corticosterone levels of both exposed and sham-exposed animals from the preterminal to the terminal period was significant on the basis of a paired t-test of the combined data from all animals from both treatment groups contributing data to both sampling periods (t = 2.79, p = .0117, df = 19). This finding supports the conclusion that the procedural change accompanying the final kill was mildly stressful. Although the mean corticosterone level during the terminal sampling period was lower for the exposed group than for the sham-exposed group, the difference was nonsignificant (t = -1.64, p = .11, df = 21). Similarly, the mean difference between the preterminal and terminal measures for the exposed animals was not significantly different from that of the sham-exposed animals (t = -1.46, p = .16, df = 18). A positive correlation was found between the preterminal and terminal corticosterone levels for the sham-exposed animals (r = .4653), but little correlation for the exposed animals (r = .006). This difference is interesting; however, because of the large variation associated with the data, the correlations were possibly not well determined.

Discussion

Chronic individual housing has been reported to lead to elevations of corticosterone indicative of a highly stressed animal, termed the "isolation-stress syndrome" (Ader, 1965; Hatch et al., 1965; Lovely et al., 1972). Although procedural limitations precluded our obtaining corticosterone measures truly reflective of basal conditions, comparison of our values to those reported in the literature indicate the absence of highly elevated mean levels despite the extended period of individual housing experienced by both the exposed and sham-exposed populations.

The absence of elevated corticosterone levels has been noted in previous studies in which the waveguide exposure apparatus was used (Moe et al., 1977; Lovely et al., 1977, 1978b; Johnson et al., 1978). One noticeable procedural difference between the isolation studies reported in the literature and the waveguide studies is that the waveguide-system maintenance schedule requires twice-daily handling of the animals as the apparatus is cleaned. This handling may have acted as a mechanism of socialization and gentling, known to reduce pituitary-adrenal reactivity

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(Ruegamer and Silverman, 1956; Barrett and Stockham, 1963; Hodges and Mitchley, 1970; Levine and Coover, 1976). Also, the waveguide may provide a protected thermal environment for animals, blocking direct air currents through the cage. The normal laboratory caging is of the hanging-metal design, which provides the animals little protection against drafts.

Analysis of the data obtained during the five sampling periods indicates that serum corticosterone levels were not dramatically altered in either the exposed or sham-exposed groups. The multivariate statist.cal analyses of the data indicates that no overall cumulative effects of microwave radiation were measurable by levels of serum corticosterone.

When the serum corticosterone values of the exposed and sham-exposed groups were compared, the t-test analysis indicated that the exposed animals may have had elevated serum corticosterone values at the time of the first sampling session and the sham-exposed animals may have had elevated levels at the time of session three. The exposed and sham-exposed groups had comparable levels of corticosterone on all other regular sampling sessions.

Rats exposed chronically or repeatedly to a stressful stimulus or environment respond with an initial period of elevated corticosterone levels followed by an adaptation period that is characterized by a return to baseline corticosterone levels despite continued exposure to the stressor (Selye, 1946; Yuwiler, 1971). This adaptation is not simply the dismissal of the stimulus as no longer stressful but is an active inhibition of response (Riegle, 1973; Daniels-Severs et al., 1973; Burchfield et al., 1980). This inhibitory process can be interpreted as an evolutionarily adaptive response mechanism that provides for physiological conservation and homeostasis (Engel and Schmale, 1972).

Adaptation is not synonymous with a return to normal functioning of the pituitary-adrenal system. Evidence indicates that the system becomes hypersensitive in its response to the disinhibiting effects of novel acute stressors (Sakellaris and Vernikos-Danellis, 1974, 1975).

Chronic stress eventually leads to what Selye terms "exhaustion," i.e., the inability to respond physiologically to stressful stimuli. Specifically, this inability has been demonstrated in aging rats; the baseline pituitary-adrenal-system functioning remained intact, but the reserve capacity to respond to stress was diminished (Barnett and Phillips, 1976; Hess et al., 1970; Hess and Riegle, 1970).

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CONCLUSIONS

Except for during the first assessment period, open-field activity and corticosterone levels were not significantly altered by 2 years of exposure to low-level pulsed-microwave radiation.

The elevation of corticosterone during the first assessment period, the return to control levels throughout the remaining periods, and the decrement in activity by the exposed animals during the first open-field assessment, together with subsequent comparability between treatment groups, are not inconsistent with a tentative conclusion that microwave exposure may have been minimally stressful.

Although additional studies are needed to verify these findings, the tentative hypothesis suggested by these data may be valuable in the interpretation of immunological, metabolic, and histological changes discussed in subsequent reports. After all subtopics have been discussed, evaluation of the physiological significance of these data will be presented in Volume 9 of this series.

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